

DUAL TESTING FOR THE SERODIAGNOSIS OF SYPHILIS

AN ABBREVIATED COMPLEMENT-FIXATION METHOD

BY

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Since the publication of the original complement-fixation test for syphilis by Wassermann, Neisser, Bruck, and Schucht (1906) and the publication of the flocculation test of Porges and Meier (1908), innumerable modifications have been described for the improvement of both diagnostic methods. The technical complexity of the former furnished an incentive for the development of the latter. Yet, despite the daily titrations and adjustment of reagents, and despite the minute measurements required, the complement-fixation test was easy to read; whereas the easily assembled flocculation tests were difficult to read and, often, more difficult to interpret. It is, however, probable that had a good flocculation test been first in the field, the complement-fixation test for syphilis would never have achieved more than academic recognition as an amazing reaction involving the tissues and body-fluids of ox, sheep, rabbit, guinea-pig, and syphilitic man.

From all the attempts that have been made to perfect the specificity and sensitivity of alternative tests to the Wassermann, there has emerged the conviction that, for the serodiagnosis of syphilis, no single test suffices, but that confidence is most wisely placed in an opinion based upon the results of both a flocculation and a complement-fixation test.

The dual control is necessitated, primarily, by a phenomenon characteristic of all flocculation tests. Although generally admitted to be more sensitive than the complement-fixation test, they exhibit, on occasion, the so-called "prozone reaction". This phenomenon occurs when the antibody present greatly exceeds in amount the antigen used. The antigen-antibody reaction is inhibited or obscured and the test fails. The prozone reaction is never encountered in the complement-fixation test, provided the serum volume is small (0.05 ml. to 0.1 ml.), but can be demonstrated by increasing the serum volume to 0.5 ml.

The proportion of sera exhibiting a prozone reaction in the standard flocculation test used in this laboratory (Ford Robertson and Colquhoun, 1939) is extremely small. In a survey by Colquhoun, Kyles, and Rannie (1945) extending over a period of 16 months, using in full both the standard flocculation and Wassermann tests, 8,342 sera were investigated. Of this number, 26 yielded a negative result in the flocculation test and a positive result in the complement-fixation test. Both tests showed agreement in 6,853 cases.

The fact that such discrepancies *can* occur is the most cogent argument in favour of including a complement-fixation test as one of those employed in the serodiagnosis of syphilis. The present-day use of extremely sensitive flocculation tests as "screen" tests can only result in repeated failures to detect prozone cases and is, therefore, indefensible.

Notice must also be taken of those cases in which the flocculation test yields a positive and the complement-fixation test a completely negative result. In the survey referred to this occurred in 51 cases.

When, for these reasons, it is thought desirable to submit large numbers of sera to both flocculation and complement-fixation tests, it becomes a matter of urgency to study ways and means of eliminating unnecessary work and of expediting essential procedures. Further, although, in serological work great speed is not conducive to accuracy, fatigue and tedium are even more inimical to precision. The following outline of a system of performing both flocculation and complement-fixation tests on the same sera is not complete: its purpose is to describe time- and fatigue-saving procedures that have yielded excellent results over a long period.

Technique

The main features of the system are:

- (1) the use of an "abbreviated" Wassermann test,
- (2) the combination of reagents common to particular sections of the Wassermann test,

- (3) a rational system of handling samples and distributing sera.

(1) Abbreviated Wassermann Test.—On the day before the Wassermann test is to be performed, all sera are submitted to the standard flocculation test. The test is read on the following morning and all positive and doubtful sera are submitted to the *full* Wassermann test. All other sera are submitted to the abbreviated test.

The full test consists of three serum-test tubes and a serum-control tube, containing 2, 4, 6, and 2 M.H.D. of complement respectively. In the abbreviated test the 4 and 6 M.H.D. tubes are omitted. While serving to eliminate completely the dangers associated with prozone reactions, the abbreviation described effects a considerable saving of time and material, inasmuch as about 80 per cent. of all sera submitted for routine examination are flocculation-test-negative. In the survey by Colquhoun and others (1945), had the abbreviated test been applied as here described, the technical labour would have been reduced by 13,905 tubes, and 69,530 doses of complement would have been saved without any sacrifice of useful information.

(2) Combination of Reagents.—The distribution of undiluted complement in volumes of the order of 0.01 ml. demands extreme accuracy of measurement and nicety of standardization. Failure to add complement to a first-row tube will be reflected, in the result, by an apparent weak-positive reaction.

Harrison and Wyler (1929) and other workers incorporate the complement in saline to standard volumes of mixture which are added to all tubes, after the distribution, to test and control tubes respectively, of serum and saline.

It has been found convenient to incorporate the complement in the antigen suspension for addition to the serum-test tubes and in saline for addition to the serum control tubes, so that in every case a single standard addition of 0.5 ml. of the appropriate reagent mixture is made. Using the method of pipetting described later, a skilled worker can make the correct addition of reagent mixture to fifty tubes in two minutes.

(3) Distribution of Sera.—The method of handling sera and reagents that has proved most expeditious will be apparent from the following outline procedure for classifying sera by the standard flocculation test and conducting the Wassermann test.

Method

Specimens.—Blood specimens are received for test in 1 oz., wide-mouthed, screw-capped bottles.* The wide mouth facilitates removal of the blood clot.

* These have been specially made for this laboratory by the United Glass Bottle Manufacturers Company.

First Day.

Distribution of Sera.—Specimen bottles are assembled in racks holding six rows of ten and are numbered serially.

With a 1-ml. graduated pipette, furnished with rubber tube and quill glass mouthpiece, 0.9 ml. of serum is withdrawn from a specimen bottle. Into the appropriate tubes of the standard flocculation test are delivered 0.3 ml., 0.2 ml., and 0.1 ml. volumes of serum. The remaining 0.3 ml. is delivered into a 3 in. \times $\frac{1}{2}$ in. tube bearing the same serial number as the specimen bottle from which the serum was withdrawn and held in a metal rack accommodating six rows of ten tubes.

For ease in reading, the pipettes should be marked with a grease pencil at the 0.9-ml., 0.6-ml., 0.4-ml., and 0.3-ml. graduations. After each specimen has been distributed, the pipette is washed out thrice with 0.85 per cent. saline. The rack containing the 0.3-ml. volumes of serum is placed in the refrigerator together with the original specimen bottles. The standard flocculation test is then set up.

Second Day.

Complement Titration.—The method is that described by Browning and MacKenzie (1924). A 3 per cent. red-blood-cell suspension, standardized by packed cell volume determination, is sensitized with 5 M.H.D. of immune body. One volume of complement is diluted with three volumes of 0.85 per cent. saline, and 0.01 ml., 0.02 ml., 0.03 ml., 0.04 ml., 0.05 ml., and 0.06 ml. volumes of the mixture are pipetted into size 3 in. \times $\frac{1}{2}$ in. tubes. To each tube are added 0.5 ml. 0.85 per cent. saline and 0.5 ml. sensitized red-blood-cell suspension. The tubes are incubated in a water bath for one hour, at 37° C. and the test read.

During the incubation period the flocculation test results are read and recorded, and the sera for the complement-fixation test are taken from the refrigerator and inactivated by heating in a water bath at 56° C. for 20 minutes.

Setting Up the Wassermann Test.—The racks, holding four rows of ten 3 in. \times $\frac{1}{2}$ in. tubes, are arranged end to end in a single row on the working bench. Each column of four holes is numbered serially from left to right to correspond to the numbering of the sera tested.

The tubes in the first (front) row each receive 2 M.H.D. of complement, those in the second row 4 M.H.D., and those in the third row 6 M.H.D. The tubes in the fourth (back) row receive 2 M.H.D. of complement: these are the serum control tubes.

In preparing for the Wassermann test, flocculation-test-positive and doubtful sera are allotted four tubes. The remaining sera are allotted two tubes; one in the front row and one in the back row.

Distribution of the Wassermann Test Sera.—A 0.2-ml. graduated pipette is used and is always filled with serum to the 0.2-ml. mark.

After each serum has been distributed the pipette is rinsed out thrice with 0.85 per cent. saline.

All tubes receive 0.05 ml. serum. The excess 0.1 ml. of a two-tube-test serum is discarded before rinsing the pipette.

The above procedure renders it impossible to pipette the same serum twice. Also, if a serum has not been pipetted the omission will be immediately apparent from the volume of that serum in the inactivating tube, viz., 0.3 ml. as compared with the residual 0.1 ml. of sera that have been pipetted.

Antigen Suspension.—The following example illustrates the necessary calculations.

Of 58 sera submitted to the flocculation test, ten have yielded a positive or doubtful reaction. In the abbreviated test, therefore, antigen suspension will be required for $(58 + 10 + 10) = 78$ tubes. To allow a "working margin", antigen suspension must be prepared for a larger number of tubes. Convenient proportions of alcoholic antigen to saline are 1.5 ml. of the former to 43.5 ml. of the latter, yielding sufficient suspension for ninety tubes.

Complement.—Antigen-complement mixtures are prepared for each of the first three rows of tubes so that the tubes in each row shall receive the appropriate dose of complement. The following Table illustrates the calculations required.

TABLE

Row	No. of tubes in row +2	Vol. of undiluted g. p. serum required	Vol. of antigen suspension (or saline) required	Total vol. of reagent mixture
First 2 M.H.D.	60	0.01 ml. \times 60 = 0.6 ml.	29.4 ml.	30.0 ml.
Second 4 M.H.D.	12	0.02 ml. \times 12 = 0.24 ml.	5.76 ml.	6.0 ml.
Third 6 M.H.D.	12	0.03 ml. \times 12 = 0.36 ml.	5.64 ml.	6.0 ml.
Fourth 2 M.H.D.	60	0.01 ml. \times 60 = 0.6 ml.	Saline 29.4 ml.	30.0 ml.

Complement dose: 1 M.H.D. = 0.005 ml. undiluted serum.

The calculation for the fourth row should be performed, and not assumed to be the same as that for the first row. In this way the quantities of reagent used in the two most critical sections of the test receive a double check.

Distribution of Reagent Mixtures.—Antigen-complement mixtures, saline-complement mixture, and sensitized red-blood-cell suspension are all added in 0.5 ml. volumes. As there are always ten tubes in the first and fourth rows of each rack (except, as a rule, the last) exactly 5 ml. of reagent are required for each.

The reagents are added from 5-ml. graduated pipettes furnished with rubber tube and quill glass mouthpiece. Each 0.5-ml. interval is marked with grease pencil in order to facilitate reading. The fact that one filling of the pipette suffices exactly to supply the appropriate reagents to the first and fourth rows of tubes in each rack (except, perhaps, the last) provides a first check upon the correctness of the additions.

Saline-complement mixture is first added to the tubes in the fourth row. The appropriate antigen-complement mixtures are then added to the first, second, and third row tubes in that order. Each rack is shaken and placed in a water bath at 37° C. for 1½ hours. At the end of this period the sensitized red-blood-cell suspension is added to every tube. When the racks have been shaken thoroughly they are returned to the 37° C. water bath for a period of 1 hour, after which the test is read.

Summary

Results obtained by modern, highly-sensitive flocculation tests for syphilis should be checked by submitting the sera to a subsequent, abbreviated complement-fixation test.

Time- and labour- saving devices, e.g. incorporation of antigen and complement in a single reagent mixture, are discussed.

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APPENDIX

PREPARATION AND STANDARDIZATION OF ALCOHOLIC ANTIGEN EXTRACT

Acetone-dried, finely-powdered bullock heart is extracted thrice with ethyl ether ("AnalaR"). Ten grammes of the exhausted powder is extracted for 10 days with 100 ml. ethyl alcohol ("AnalaR" or equivalent) and filtered. The filtrate is made up

to 100 ml. with ethyl alcohol and 0.2 g. cholesterol added. Solution of the cholesterol is effected by warming the mixture on a water bath at 55° C. For diluting the antigen a 0.2 per cent. alcoholic solution of cholesterol is used.

Dilutions of the extract are prepared as follows :

Extract	1.0	0.8	0.6	0.4	0.2 ml.
0.2 per cent. alcoholic cholesterol	0.0	0.2	0.4	0.6	0.8 ml.

One volume of each of the above dilutions is added to 29 volumes of 0.85 per cent. saline, the mixtures are allowed to stand for 15 minutes, and their antigenic properties are tested against known weak-positive and negative sera.

In the neighbourhood of the optimum lipoid concentration, the antigen mixtures present a "smoky" or "shot silk" appearance. In antigen mixtures which remain clear the lipoid concentration is too high; they are always insensitive and need not be tested.

The optimum dilution having been found, the bulk of the cholesterinized extract is diluted accordingly. The product keeps indefinitely.